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An evaluation of the applicability of microarrays for monitoring toxic algae in Irish coastal waters

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Abstract The applicability of microarrays to monitor harmful algae across a broad range of ecological niches and toxic species responsible for harmful algal events has been one of the key tasks in the EU Seventh Framework Programme (FP7)-funded Microarrays for the Detection of Toxic Algae project. The technique has a strong potential for improving speed and accuracy of the identification of harmful algae and their toxins to assist monitoring programmes. Water samples were collected from a number of coastal sites around Ireland, including several that are used in the Irish National Phytoplankton and Biotoxin Monitoring Programme. Ribosomal RNA was extracted from filtered field samples, labelled with a fluorescent dye, and hybridised to probes spotted in a microarray format on a glass slide. The fluorescent signal intensity of the hybridisation to >120 probes on the chip was analysed and compared with actual field counts. There was a general agreement between cell counts and microarray signal. Results are presented for field samples taken from a range of stations along the Irish coastline known for harmful algal events during the first field trial (July 2009–April 2010).

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Introduction

Blooms of toxic or harmful microalgae, commonly referred to as harmful algal blooms (HABs), represent a significant threat to fisheries resources and human health throughout the world. These HABs manifest themselves in many ways, ranging from high phytoplankton biomass that discolours seawater and reduce water quality, to low-cell density but highly toxic populations which can contaminate shellfish (GEOHAB 2001). The aquaculture industry in Ireland is a valuable resource and has been estimated to be worth approximately €60 million annually to the Irish economy (Browne et al. 2007). Monitoring programmes have become a necessity because of the potential dangers to human health and the significant economic impacts of contaminated seafood posed by harmful events. In Europe, this requirement for monitoring is established in a series of directives in which monitoring of coastal waters for the presence of potentially harmful phytoplankton is mandatory (Council Directive 91/492).

Traditionally, phytoplankton monitoring has been carried out by identification and enumeration using light microscopy. It has been recognised for some time that this technique requires a high degree of skill on behalf of the operator and is time consuming (Penna et al. 2007; Karlson et al. 2010). Furthermore, the morphological similarity between different species within or even across phytoplankton genera has meant that light microscopy alone is often insufficient to assess the potential toxicity of water. A variety of identification methods based on the sequencing of nucleic acids has been developed over the past decade or so that have considerably improved our ability to accurately identify organisms to species level

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(Karlson et al. 2010). DNA-based molecular probe methods, such as fluorescent in situ hybridisation analysis (FISH), have been developed that can identify and quantify specific species in complex phytoplankton communities (Scholin et al. 1997; Scholin and Anderson 1998; Touzet et al. 2010). Utilisation of microsatellites is another molecular technique that is now becoming a popular genotyping method to answer ecological questions (Evans et al. 2004; Masseret et al. 2009). Further advances have led to the development of DNA biosensors for electrochemical detection of phytoplankton and their toxins (Metfies et al. 2005; Campàs et al. 2007; Vilariño et al. 2009) and real-time quantitative PCR techniques which can provide accurate and reproducible quantification of gene copies (Galluzzi et al. 2008; Touzet et al. 2009; Kavanagh et al. 2010).

Microarrays are the state-of-the-art technology in molecular biology for the processing of bulk samples for the detection of target RNA/DNA sequences. They are essentially a glass microscope slide with specific RNA/DNA sequence probes spotted on the surface. Each spot is complementary to an extracted target (RNA or DNA) through the process of hybridisation. The addition of a fluorescent label to the extracted target prior to hybridisation provides the ability to measure the amount of target in the sample using a microarray scanner (Metfies and Medlin 2008). One of the first DNA microchips involved in the study of microbial diversity was used to analyse nitrifying bacteria (Guschin et al. 1997). In the present study, existing rRNA probes (18S, 28S) and antibodies for algal toxins have been adapted and optimised for microarray format in order to develop a monitoring technique that strengthens our ability to monitor bulk water samples for toxic algae. The purpose was to provide a rapid test to aid national monitoring agencies by providing new rapid tools for the identification and enumeration of toxic algae and their toxins so that they can comply with EC directive 91/492. This paper presents the results from the first year of trials with the microarray on samples taken from a range of Irish coastal stations which have had a history of harmful algal events.

Materials and methods

Algal cultures

Prymnesium parvum N.Carter, *Prymnesium polylepis* (Manton & Parke) Edvardsen, Eikrem & Probert, and *Dunaliella tertiolecta* Butcher strains were purchased from Provasoli–Guillard National Center for Culture of Marine Phytoplankton (USA), Bigelow Laboratory for Ocean Sciences, Culture Collection of Algae (Sammlung von Algenkulturen der Universität Göttingen, SAG) or kindly provided by Bente Edvardsen (Department of Biology, University of Oslo, Oslo; Table 1).

Table 1 Algal cultures used during the study

Culture collection	Strain code	Species name
University of Oslo	UIO 226 (DunA; CCAP19/6B)	*D. tertiolecta*
Provasoli-Guillard	CCMP 1757	*P. polylepis*
University of Oslo	UIO038	*P. polylepis*
University of Oslo	UIO036	*P. polylepis*
University of Oslo	UIO054 (=RHpat89)	*P. parvum*
Provasoli-Guillard	CCMP 709	*P. parvum*
SAG	SAG 127.79	*P. parvum*

CCMP Provasoli–Guillard National Center for Culture of Marine Phytoplankton, *SAG* Sammlung von Algenkulturen der Universität Göttingen

Algal cultures were maintained in IMR½ algal medium (without silicate and with selenite; as outlined in Eppley et al. 1967, modified by E. Paasche, UiO) at 15±1 °C, under a white fluorescent light with a photon flux of 100 μE $m^{-2}s^{-1}$ and a 14:10 light/dark cycle. When required, concentrations of cells in subsamples used for calibration were enumerated using a Fuchs–Rosenthal chamber.

Field sampling

Water samples were taken from a number of coastal sites along the Irish coast. These sites included the North Channel of Cork Harbour, Bell Harbour and Lough Murray of Galway Bay, Killary Harbour, Clew Bay, Killala Bay and two sampling stations off the south west coast of Ireland (Fig. 1). At least one of the sampling stations shown in Fig. 1 was sampled every month. Water samples were prefiltered through a 150 μm mesh prior to filtration through 1 μm pore size nitrocellulose filters (25 mm diameter). The actual volume filtered depended on the turbidity of the water: 0.2–1 L was filtered up to the point when the filter started to clog. The filter was then immediately submersed in 1 ml of Tri-Reagent (Ambion, UK) within 2-ml screw cap tubes containing an aliquot of *D. tertiolecta* (5×10^6 cells) added as an internal control for the RNA extraction process. The tubes were then stored at −80 °C. For phytoplankton cell enumeration, a sample volume of 50 ml was fixed with Lugol's iodine solution (Throndsen 1978) and stored in the dark. Enumeration was carried out using an inverted microscope after settlement for 24 h in either 25 ml Utermöhl chambers or in the original 50 ml cell culture bottle and concentrations calculated accordingly (Hasle 1978; McDermott and Raine 2010).

RNA isolation

Field samples contained in the 2 ml tubes were thawed on ice and the cells removed from the filter through the addition

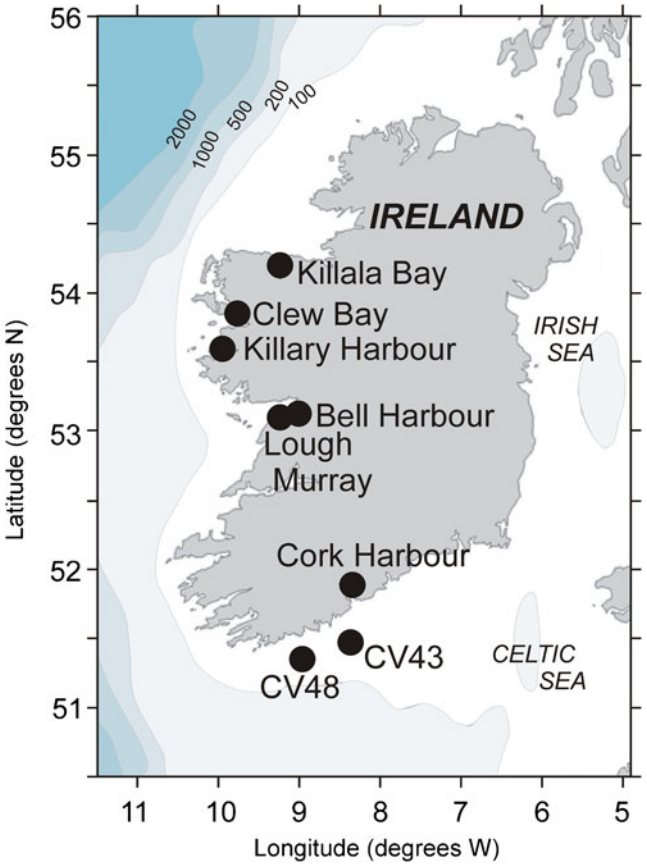


Fig. 1 Location of sites sampled during 2009–2010 along the Irish coastline

of 100 μl of 212–300 μm acid washed glass beads (Sigma-Aldrich) and heating on a thermoshaker at 60 °C for 10 min, shaking at maximum speed. The samples were periodically ribolyzed for 20 s during this treatment. There followed a sequential extraction using 1-bromo-3-chloro-propane (BCP; Sigma) and isopropanol (Sigma). An aliquot (100 μl) of BCP was added to the sample, the mixture vortexed for 15 s and transferred to prespinned 2 ml heavy phase lock tubes (5-PRIME; 12,000×*g* for 30 s), which were then shaken (by hand) for a 15 s and then allowed stand for 5 min at room temperature. The tubes were centrifuged at 4 °C for 15 min at 12,000×*g* and the supernatant layer transferred to a clean 1.5 ml RNase-free tube. An equal volume of isopropanol was added (500 μl) and the tube was vortexed for 15 s. The tube was then kept at −20 °C for 1 h, centrifuged again for 15 min, and the supernatant carefully removed using a micropipette. The RNA pellet was washed with 1 ml of 75 % ethanol, centrifuged, and the supernatant was completely removed. After the final centrifugation step, the pellet was air dried for 3–5 min and suspended in 50–100 μl RNase-free water by repeated flicking and vortexing. Nucleic acid concentrations in the sample were measured with a NanoDrop 1000 Spectrophotometer and the extract was stored at −80 °C.

RNA extraction efficiency

The efficiency of the RNA extraction method was determined by using species-specific probes for *D. tertiolecta* that had been spotted onto the microarray and acted as controls. Linearity of the extraction was investigated by extracting RNA from increasing cell numbers of *D. tertiolecta* culture carried out in triplicate, with quantification of the RNA concentration estimated using the NanoDrop 1000 Spectrophotometer. The RNA extraction of samples was also checked by preparing extractions from field samples alongside duplicate extracts to which 5×10^6 cells of *D. tertiolecta* had been spiked. These were run against extracts of 5×10^6 cells *D. tertiolecta* alone.

RNA labelling and fragmentation

The RNA (1 μg) was labelled using a Platinum Bright 647 Infrared Nucleic Acid labelling kit (KREATECH Biotechnology) according to the manufacturer's instructions. The concentration of labelled dye was measured by NanoDrop (Microarray) and the degree of labelling was subsequently calculated. Fragmentation of the labelled RNA was carried out by adding 1/10 fragmentation buffer (100 mM ZnCl2 in 100 mM Tris–HCl, pH7), incubation in a thermoshaker for 15 min at 70 °C, and the reaction was then stopped by adding 1/10 stop buffer (0.5 M EDTA, pH8) and placing samples on ice (Lewis et al. 2012).

Internal control (TBP-Cy5) preparation

DNA from bread yeast powder (*Saccharomyces cerevisiae*) was extracted using Qiagen RNeasy Plant Mini Kit according to manufacturer's instructions. The PCR cycle and primers TATA-box binding protein gene (TBP)-F (5′-ATG GCC GAT GAG GAA CGT TTA A-3′) and TBP-R_CY5 (5′-TTT TCA GAT CTA ACC TGC ACC C-3′) were used to amplify the TBP using the detailed procedure in the MIcroarrays for the Detection of Toxic ALgae (MIDTAL) Manual (Lewis et al. 2012). The PCR program was as follows: initial step of 95 °C for 5 min, 40 cycle step (95 °C 1 min, 53 °C 1 min, and 72 °C 2 min) and final step 72 °C for 5 min. The final PCR product was purified using the PCR MinElute Cleanup Kit (QIAGEN) and quantified with a Nanodrop (Microarray) and stored at −80 °C.

Probe development

Probes were initially taken from those designed for FISH detection tested in a first generation microarray and modified to extend their length as the 18 base pair oligonucleotides routinely used for FISH hybridisation were too short to achieve specific hybridisation reactions in a microarray

format. Details are provided in Table 2 but the sequences are patent pending and are not shown. Where possible the original FISH probe that was modified is indicated.

Microarray hybridisation

Details of the microarray chip development can be found in Lewis et al. (2012). Epoxysilane-coated microarray chips were prespotted with over 120 oligonucleotide probes for a range of potentially harmful phytoplankton species. The chip was prehybridised with 20 ml prehybridisation buffer (2 M NaCl; 20 mM Tris-Cl, pH8.0; 0.01 % Triton 100) for 60 min at a hybridisation temperature of 65 °C. Slides were washed with deionised water and dried using centrifugation in slide holders for 3 min at 1,800 rpm. A mixture of 35 μl 2× hybridisation buffer (1 mg/mL BSA, 0.2 μg/μL herring sperm DNA, 2 M NaCl, 20 mM Tris-Cl, pH 8.0, 0.01 % Triton 100; Lewis et al. 2012) containing the labelled RNA (1 μg) sample and 100 ng of the TATA-box positive control (TBP control) was prepared and made up to a final volume of 70 μl with RNase-free water. The hybridisation mixture was then incubated at 94 °C for 5 min to denature the target labelled nucleic acid. MicroArray mSeries LifterSlips (20× 25 mm; Thermo Scientific) were placed on the microarray and half (35 μl) of the hybridisation mixture was added in duplicate to the microarray. Hybridisation was carried out at 65 °C for 1 h in a wet chamber comprising wet Whatman filter paper in a screw capped 50 ml centrifuge tube (Falcon). After 1 h, the cover slips were removed off the array and the hybridised chip surface underwent three washing buffer steps for 10 min with increasing stringency involving EDTA at room temperature, thereby minimising background noise (Lewis et al. 2012). The chip, prespotted with over 120 oligonucleotide probes corresponding to a taxonomic hierarchy (kingdom, class, genus, and species) for harmful algal species, was then scanned (Genepix 4000B Axon Inc.) and the fluorescence signal intensity from each probe was measured.

Scanning and analysis

The microarray chip was scanned using a Perkin Elmer Microarray Scanner. This output (.tiff files) were then uploaded into GenePix 6.0 software programme and with the aid of an uploaded gal file (midtal_ver252_20100423.gal) which is a gridded map corresponding to each individual probe spotted onto the microarray chip, the fluorescent signals and background intensities could be calculated for each probe. The results were then saved as a separate GPR file which was imported into the PhylochipAnalyzer graphical Windows programme or GPR-analyser (gpr-analyzer ver 1.25), which allows description of the hierarchy of the probe set (Dittami and Edvardsen 2012). The signal-to-noise ratio of 2.0 was set as a cutoff for positive signals. Total signal intensities were normalised against the positive controls (Poly-T-CY5, Positive_25 and DunGS02_25) to quantify results from different hybridisations. Microarray results were then compared with light microscopic counts of the original water samples. This was carried out on samples taken over a period of 1 year.

Results and discussion

RNA extraction efficiency

The relationship between cell numbers of *D. tertiolecta* and the amount of RNA extracted was linear with a satisfactory coefficient of determination (R^2=0.9243, n=14; Fig. 2). Figure 3 shows a comparison between RNA extractions from field samples with and without spiking of 5×10^6 cells of *D. tertiolecta*. The RNA extraction efficiency was satisfactory because the average difference between spiked and unspiked samples (mean=9,800, ±2,600 ng) was very similar to the average RNA amount (mean=11,000, ±1,400 ng) extracted from parallel *D. teriolecta* controls. This validated the nucleic acid extraction method, making it suitable for quantification in both laboratory studies and in situ field sample analysis.

Chip development and sensitivity

The first chip designed for a specified range of HAB species produced weak signals for several species-probe combinations. A second generation chip was subsequently designed in which the probes were increased in length by up to 25 base pairs with the main aim of increasing probe specificity and decreasing cross reactivity (Fig. 4). The melting point temperature was subsequently altered to 65 °C, which was adopted as standard. Figure 4 compares data obtained from the first and second generation chips. The sample chosen was from Bell Harbour, where a bloom of *Prorocentrum micans* occurred in 2009. Examination under the light microscope (LM) showed that this was the dominant species (360,000 cells l^{-1}). The original *P. micans* probe spotted on the first generation chip (Pmica02; 37.15 s/n ratio) gave a substantial lower signal than its modified version on the second generation microarray. Cross-reactivity was also reduced using the second generation chip with a consequent increase in specificity. There was also a reduction signal from the class level probe for Dinoflagellates, Heterokonta, *Pseudo-nitzschia* species, *Dunaliella* genus level, Eukaryotes kingdom level probes, and the spotted controls Poly-T and Poly-T-CY5.

The sensitivity of the extraction procedure to hybridisations of increasing amounts of labelled RNA was investigated

Table 2 Summary of probes designed or modified from those published for FISH hybridisation and used to form the second generation of the MIDTAL microarray

Probe name	Targeted species	Gene	Tm (GC% method)	Source/DESIGNER
Higher group probes				
EukS_328_25	Eukaryotes	18S	79	Moon-van der Staay et al. (2001)
EukS_1209_25	Eukaryotes	18S	79	Lim et al. (1993)
HeteroS01_25	Heterokonta	18S	77.3	Eller et al. (2007)
PrymS01_25	Prymnesiophyta	18S	77.3	Lange et al. (1996)
PrymS02_25	Prymnesiophyta	18S	80.6	Simon et al. (2000)
PrymS03_25	Prymnesiophyceae	18S	77.3	Eller et al. (2007)
Class Level Probes				
DinoB_25	Dinophyceae (incl. Apicomplexa)	18S	75.7	John et al. (2003)
DinoE12_25	Dinophyceae (incl. Apicomplexa)	18S	77.3	Groben, John and Medlin, (unpublished)
ProroFD01	Prorocentrum clade	28S	77.3	Groben, Lange and Medlin (unpublished)
DphyFS01_25	Dinophysiaceae (Dinophysis+Phalacroma)	18S	77.3	Edvardsen, Groben, Brubak and Medlin (unpublished)
DphyFS02_25	Dinophysiaceae (Dinophysis+Phalacroma)	18S	79	Edvardsen, Groben, Brubak and Medlin (unpublished)
Genus Level Probes				
PrymGS01_25	Prymnesium	18S	79	Eller et al. (2007)
PrymGS02_25	Prymnesium	18S	79	Eller et al. (2007)
PsnGS01_25	Pseudo-nitzschia	18S	77.3	Eller et al. (2007)
PsnGS02_25	Pseudo-nitzschia	18S	79	Eller et al. (2007)
KareGD01_25	Karenia	28S	77.4	MIDTAL project
AlexGD01_25	Alexandrium	28S	75.7	MIDTAL project
DphyGD01	Dinophysis in part	28S	77.3	Guillou et al. (2002)
DphyGD02	Dinophysis	28S	75.6	Guillou et al. (2002)
PschGS01_25	Pseudochattonella (genus)	18S	77.3	MIDTAL project
PschGS02_25	Pseudochattonella (genus)	18S		MIDTAL project
PschGS03_25	Pseudochattonella (genus)	18S		MIDTAL project
PschGS04_25	Pseudochattonella (genus)	18S	77.3	MIDTAL project
PschG05_25	Pseudochattonella (genus)	18S	79	MIDTAL project
DphyGS01_25	Dinophysis genus sensu stricto	18S	75.9	Edvardsen, Groben, Brubak and Medlin (unpublished)
DphyGS02_25	Dinophysis genus sensu stricto	18S	79	Edvardsen, Groben, Brubak and Medlin (unpublished)
DphyGS03_25	All Dinophysis and Phalacroma	18S	80.6	Edvardsen, Groben, Brubak and Medlin (unpublished)
DphyGS04_25	All Dinophysis	18S	77.3	Edvardsen, Groben, Brubak and Medlin (unpublished)
PrymGS01_25	Prymnesium	18S		MIDTAL project
KargeD01_25	Karlodinium genus	28S	75.6	MIDTAL project
AzaGD01	Azadinium Genus	28S	75.9	MIDTAL project
AzaGD03	Azadinium Genus	28S	75.7	MIDTAL project
AzaGS01	Azadinium Genus	18S	79	MIDTAL project
AzaGS02	Azadinium Genus	18S	79	MIDTAL project
L*Kare0308A25	Karenia genus	28S	80.6	MIDTAL project
ProtuS01_25	Phalacroma Genus	18S	79	Edvardsen, Groben, Brubak and Medlin (unpublished)
Species Level Probes				
AtamaS01_25	Alexandrium NA,WE,TA, species complex	18S	77.3	John et al. (2003)
AminuS01_25	*A. minutum*	18S	79	Miller and Scholin (1998)
ATNA_D01_25	*A. tamarense* (North America)	28S	79	John et al. (2003)
ATNA_D02_25	*A. tamarense* (North America)	28S	77.3	Guillou et al. (2002)
ATTA _D01_25	*A. tamarense* (Temperate Asian)	28S	77.3	MIDTAL project
AostD01 _25	*A. ostenfeldii*	28S	75.7	John et al. (2003)
AostS02 _25	*A. ostenfeldii*	18S	79	John et al. (2003)

Table 2 (continued)

Probe name	Targeted species	Gene	Tm (GC% method)	Source/DESIGNER
CpolyS01_25	Prymnesium polylepis	18S	77.3	Simon et al. (1997)
PparvD01_25	*P. parvum*	28S		Töbe et al. (2006)
KbreD03_25	*K. mikimotoi* and *brevis*	28S		Mikulski et al. (2005)
KbreD04_25	*K. mikimotoi* and *brevis*	28S	79	Mikulski et al. (2005)
KmikiD01_25	*K. mikimotoi*	28S	79	Guillou et al. (2002)
KbreD05_25	*K. brevis*	28S	80.6	Mikulski et al. (2005)
SSKbre1448A25	*K. brevis*	18S	80.6	MIDTAL project
LSKbre0548A25	*K. brevis*	28S	82.3	MIDTAL project
KveneD01_25	*Karlodinium veneficum*	28S	77.3	MIDTAL project
KveneD02_25	*K. veneficum*	28S	72.4	MIDTAL project
KveneD03_25	*K. veneficum*	28S	74.1	MIDTAL project
KveneD04_25	*K. veneficum*	28S	80.6	MIDTAL project
KveneD05_25	*K. veneficum*	28S	79	MIDTAL project
KveneD06_25	*K. veneficum*	28	75.7	MIDTAL project
PlimaS01_25	*P. lima*	18S	77.3	Groben, Lange and Medlin (unpublished)
PlimaD01_25	*P. lima*	28S	80.6	Groben, Lange and Medlin (unpublished)
PmicaD02_25	*P. micans*	28S	80.6	Groben, Lange and Medlin (unpublished)
PminiD01_25	*P. minimum*	28S	79	Groben, Lange and Medlin (unpublished)
GcateS01_25	*G. catenatum*	18S	76	Diercks et al. (2008)
DacumiD02_25	*D. acuminata*+dens+sacculus	28S	79	Guillou et al. (2002)
DacutaD02_25	*D. acuta*+*D. fortii*	28S	79	Guillou et al. (2002)
DacumiS01_25	*D. acuminata*	18S	80.6	Edvardsen, Groben, Brubak and Medlin (unpublished)
DacutaS01_25	*D. acuta*	18S	77.3	Edvardsen, Groben, Brubak and Medlin (unpublished)
DnorvS01_25	*Dinophysis norvegica*	18S	77.3	Edvardsen, Groben, Brubak and Medlin (unpublished)
ProtuS01_25	*Phalacroma rotundatum*	18S	79	Edvardsen, Groben, Brubak and Medlin (unpublished)
PaustS01_25	*P. australis*	18S	80.6	Diercks et al. (2008)
PmultS01_25	*P. multiseries*	18S	80.8	Diercks et al. (2008)
PpungS01_25	*P. pungens*	18S	79	Diercks et al. (2008)
PamerD01_25	*P. americana*	28S	79	MIDTAL project
PaustD01_25	*P. australis* and *P. multistriala*	28S	77.3	MIDTAL project
PdeliD02_25	*P. delicatissima*	28S	75.7	MIDTAL project
PfrauD02_25	*P. fraudulenta* and *P. subfraudulenta*	28S	82.3	MIDTAL project
PfrauD04_25	*P. fraudulenta*	28S	82.1	MIDTAL project
PaustD02_25	*P. australis* and *P. seriata*	28S	77.3	MIDTAL project
PaustD03_25	*P. australis* and *P. seriata*	28S	83.9	MIDTAL project
PbrasD01_25	*P. brasiliana*	28S	79	MIDTAL project
PbrasD02_25	*P. brasiliana*	28S	78.9	MIDTAL project
PbrasD03_25	*P. brasiliana*	28S	79	MIDTAL project
PcaciD01_25	*P. caciantha*	28S	74.1	MIDTAL project
PcaciD02_25	*P. caciantha*	28S	79	MIDTAL project
PcaciD04_25	*P. caciantha*	28S	75.7	MIDTAL project
Pcal1D01_25	*P. caciantha*	28S	77.3	MIDTAL project
Pcal2D01_25	*P. caciantha*	28S	77.3	MIDTAL project
Pcal2D02_25	*P. caciantha*	28S	75.7	MIDTAL project
Pcal2D03_25	*P. caciantha*	28S	77.4	MIDTAL project
Pcal2D05_25	*P. caciantha*	28S	77.4	MIDTAL project
Pdel1D01_25	*P. delicatissima*	28S	74.1	MIDTAL project
Pdel2D01_25	*P.* cf. *delicatissima* clade4	28S	79	MIDTAL project

Table 2 (continued)

Probe name	Targeted species	Gene	Tm (GC% method)	Source/DESIGNER
Pdel2D02_25	*P.* cf. *delicatissima* clade4	28S	74.1	MIDTAL project
Pdel3D01_25	*P. arenysensis*	28S	79	MIDTAL project
Pdel1D03_25	*P. delicatissima*	28S	79	MIDTAL project
PgalaD01_25	*P. galaxiae*	28S	75.8	MIDTAL project
PgalaD02_25	*P. galaxiae*	28S	75.7	MIDTAL project
PgalaD04_25	*P. galaxiae*	28S	74.1	MIDTAL project
PhemeD2_25	*P. hemeii*	28S	77.3	MIDTAL project
PmultD01 _25	*P. multiseries*	28S	80.8	MIDTAL project
PmultD02 _25	*P. multiseries*	28S	77.3	MIDTAL project
PmultD03 _25	*P. multiseries*	28S	84.2	MIDTAL project
PmultD04 _25	*P. multiseries*	28S	80.8	MIDTAL project
PmulaD02_25	*P. multistriata*	28S	81	MIDTAL project
PmulaD03_25	*P. multistriata*	28S	77.3	MIDTAL project
PpdeD01_25	*P. pseudodelicatissima* and *P. cuspidata*	28S	74.1	MIDTAL project
PpdeD02_25	*P. pseudodelicatissima* and *P. cuspidata*	28S	79	MIDTAL project
PpungD02_25	*P. pungens*	28S	82.7	MIDTAL project
PpungD04_25	*P. pungens*	28S	80.8	MIDTAL project
PsubpD01_25	*P. subpacifica*	28S	77.3	MIDTAL project
PseriD01_25	*P. seriata*	28S	79	MIDTAL project
PturgD1_25	*P. turgiduloides*	28S	74.2	MIDTAL project
PturgD3_25	*P. turgiduloides*	28S	79	MIDTAL project
Pdel4D01_25	*P.* cf. *delicatissima* clade4	28S	79	MIDTAL project
PvigoD01	*P. hasleana*	28S	79	MIDTAL project
PvigoD03	*P. hasleana*	28S	79	MIDTAL project
CtoxiS05	cf. *Chatonella* sp	18S	78.8	MIDTAL project
CtoxiS06	cf. *Chatonella* sp	18S	78.9	MIDTAL project
CtoxiS07	cf. *Chatonella* sp	18S	80.6	MIDTAL project
CtoxiS09	cf. *Chatonella* sp	18S	78.8	MIDTAL project
SSGcat0826A27	*G. catenatum*	18S	77.4	MIDTAL project
LSGcat0270A24	*G. catenatum*	28S	80.8	MIDTAL project
LSGcat0544A24	*G. catenatum*	28S	82.5	MIDTAL project
SSHaka0193A25	*Heterosigma akashiwo*	*18S*	79	MIDTAL project
SSHaka0200A25	*H. akashiwo*	18S	77.4	MIDTAL project
LSHaka0544A25b	*H. akashiwo*	28S	82.3	Tyrrell et al. (2001)
LSHaka0268A25	*H. akashiwo*	28S	82.3	Tyrrell et al. (2001)
LSHaka0544A25c	*H. akashiwo*	28S	82.3	Tyrrell et al. (2001)
LSHaka0548A25	*H. akashiwo*	28S	82.3	Chen et al. (2008)
LSHaka0329A25	*H. akashiwo*	28S	82.3	MIDTAL project
LSHaka0358A24	*H. akashiwo*	28S	82.5	Bowers et al. 2006
PfarD01_25	*Pseudochattonella farcimen*	28S	78	MIDTAL project

Probe sequences are not provided because the microarray is patent pending

by testing a range of probes that should be highlighted by a particular organism. Figure 5a and b show calibration curve results for probes designed for prymnesiophytes that were tested using a culture of *P. parvum* and *P. polylepis*, respectively. The probes were adapted for the microarray from the original sequences published by Lange et al. (1996), Simon et al. (1997, 2000), Töbe et al. (2006), and Eller et al. (2007). The RNA extract was quantified after the labelling and clean-up steps so that approximately 1, 5, 25, and 100 ng was hybridised to the chip. The performance of a series of

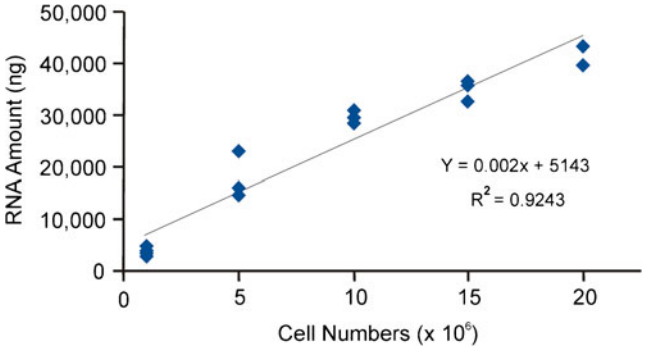


Fig. 2 Calibration curve of RNA amount against cell numbers using a culture of *D. tertiolecta* (UIO 226)

hierarchical probes is demonstrated in Fig. 5. Certain probes, also listed in Table 3, performed poorly and results from these spots were not used in the calibration or for field samples. The quantification limit was represented by a signal-to-noise ratio of 2, a value also obtained for several other probes (MIDTAL, papers in this issue). Thus, if the Prymnesiophytes probe (PrymS01_25; Lange et al., 1996) is applied, the microarray cannot measure RNA amounts below 5 ng, which is equivalent to 8,800 cells for *P. parvum* and 3,800 cells for *P. polylepis* species (Fig. 5). In order to derive cell numbers from RNA quantities, stress experiments (irradiance, nutrients, salinity, and temperature) were performed on multiple strains of *P. parvum* and *P. polylepis* cultures (Table 1). This allowed to determine the average amount of RNA per cell of *P. parvum* (mean=0.570±0.160 pg/cell) and *P. polylepis* (mean=1.331±0.674 pg/cell) over a range of environmental conditions (data not shown). An example of increasing intensities is shown in Fig. 5 with the Prymnesiophyta probe (PrymS01_25) being used with increasing amounts of *P. parvum* and *P. polylepis* RNA. This approach allowed the construction of calibration curves for each probe on the microarray chip, enabling the conversion from signal intensity to cell numbers and hence the use of the microarray for quantification purposes.

Light microscopy and microarray field results

A total of 13 samples were collected between 16.07.09 to 07.04.10 and the RNA extracted. These extracts were hybridised onto the modified (second generation) chip. Results from a selected number of probes are shown in Fig. 6.

A comparison between light microscopy counts of *Pseudo-nitzschia* spp. and microarray results from samples obtained from the 16th July 2009 to 7th April 2010 are shown in Fig. 6a–c. A total of 7 out of 13 stations samples contained *Pseudo-nitzschia* spp., classified from LM analysis either as *seriata* group (>5 μm width) or *delicatissma* group (<3 um width). In August 2009, an assemblage of *P. seriata* like organisms were numerically dominant (113, 000 cells l^{-1}) in Killala Bay. The microarray analysis detected in these samples: *Pseudo-nitzschia australis* (PaustD02_25), *Pseudo-nitzschia brasiliana* (PbrasD03_25), *P. fraudulenta* (PfrauD02_25), *Pseudo-nitzschia multiseries* (PmultD01_25), *Pseudo-nitzschia multistriata* (PmulaD03_25), *Pseudo-nitzschia pungens* (PpungS01_25, PpungD02_25, PpungD04_25), *Pseudo-*

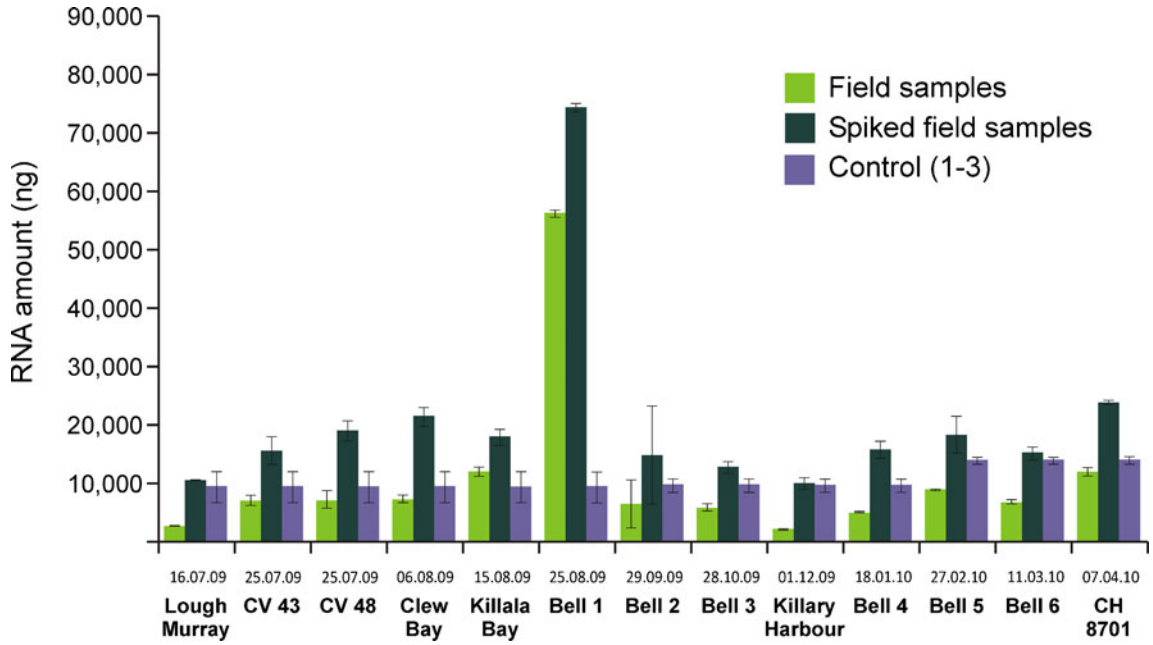


Fig. 3 RNA extraction efficiency of field samples. Extracted RNA amounts from triplicate filters with and without an internal spike (*D. tertiolecta*) are shown. Controls represent amounts of RNA extracted from known aliquots of three separate RNA extracts from pure *D. teriolecta* cultures. Three controls were (1) Jul to Aug, (2) Sept to Jan, and (3) Feb to April

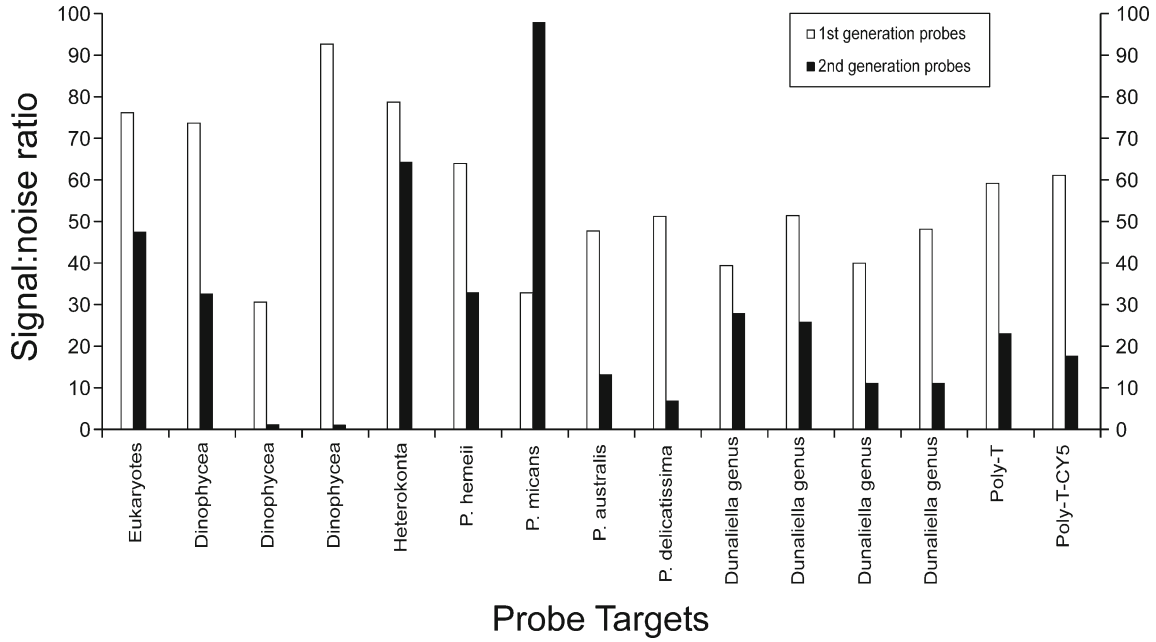


Fig. 4 Microarray results of first and second generation chips both hybridised with the same Bell Harbour field extract (25/08/09) at a temperature of 58 and 65 °C, respectively. Note the difference between signal noise ratio values in the first generation (open bar) compared with its extended second generation probe (*filled bar*)

nitzschia seriata (PseriD01_25), and *Pseudo-nitzschia subpacifica* (PsubpD01_25). In samples CV43, CV48 Clew Bay, Bell 1, Bell 3 and Bell 6 *P. seriata* group (range, 64–4,800 cells l^{-1}) were detected via LM analysis. Correspondingly in station CV43, *P. australis* (PaustD02_25), *P. brasiliana* (PbrasD03_25), *P. fraudulenta* (PfrauD02_25), *P. multiseries* (PmultD01_25), *P. pungens* (PpungS01_25, PpungD02_25, PpungD04_25), and *P. seriata* (PseriD01_25) were highlighted by the microarray with a convincing signal-to-noise ratio above 2. Samples CV48, Clew Bay, Bell 3 returned false-negative results (LM positive but microarray negative) for all the *P. seriata* group probes because the signals were below the signal-to-noise ratio threshold level of 2 (Fig. 6a and c).

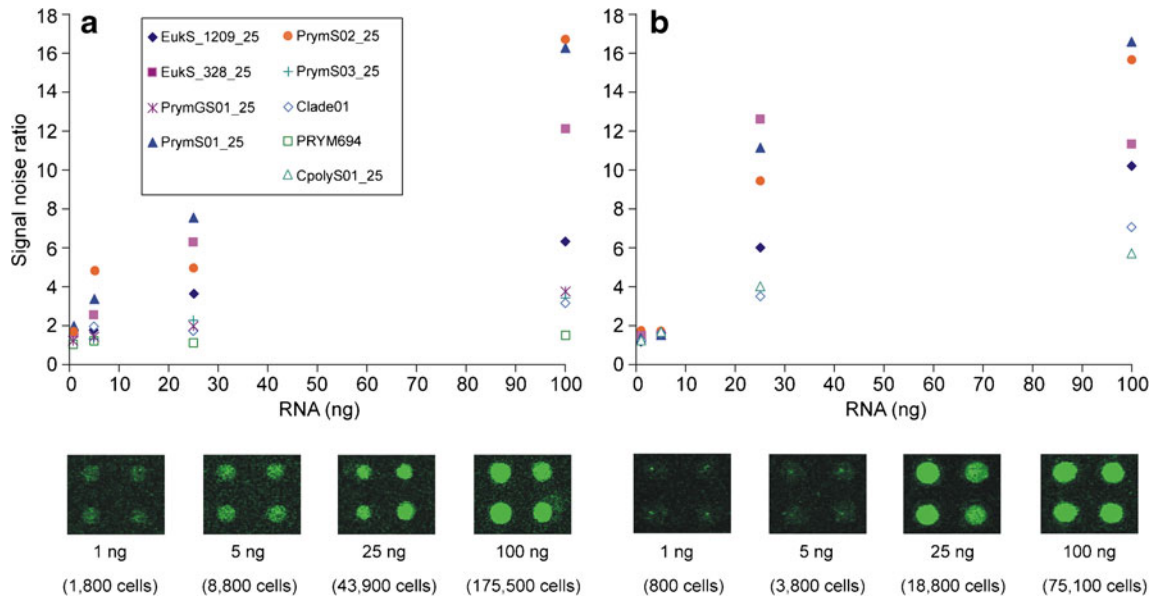


Fig. 5 Standardisation of the **a** *P. parvum* and **b** *P. polylepis* microarray signal. Calibration was carried out for 1, 5, 25, and 100 ng of RNA against signal-to-noise ratio values for a range of probes. Images are from use of the probe PrymS01_25 when increasing amounts of RNA are hybridised to the microarray. Increasing signal intensity represents increasing cell numbers

Table 3 Characteristics of calibration curves for probes targeting *P. polylepis* and *P. parvum*

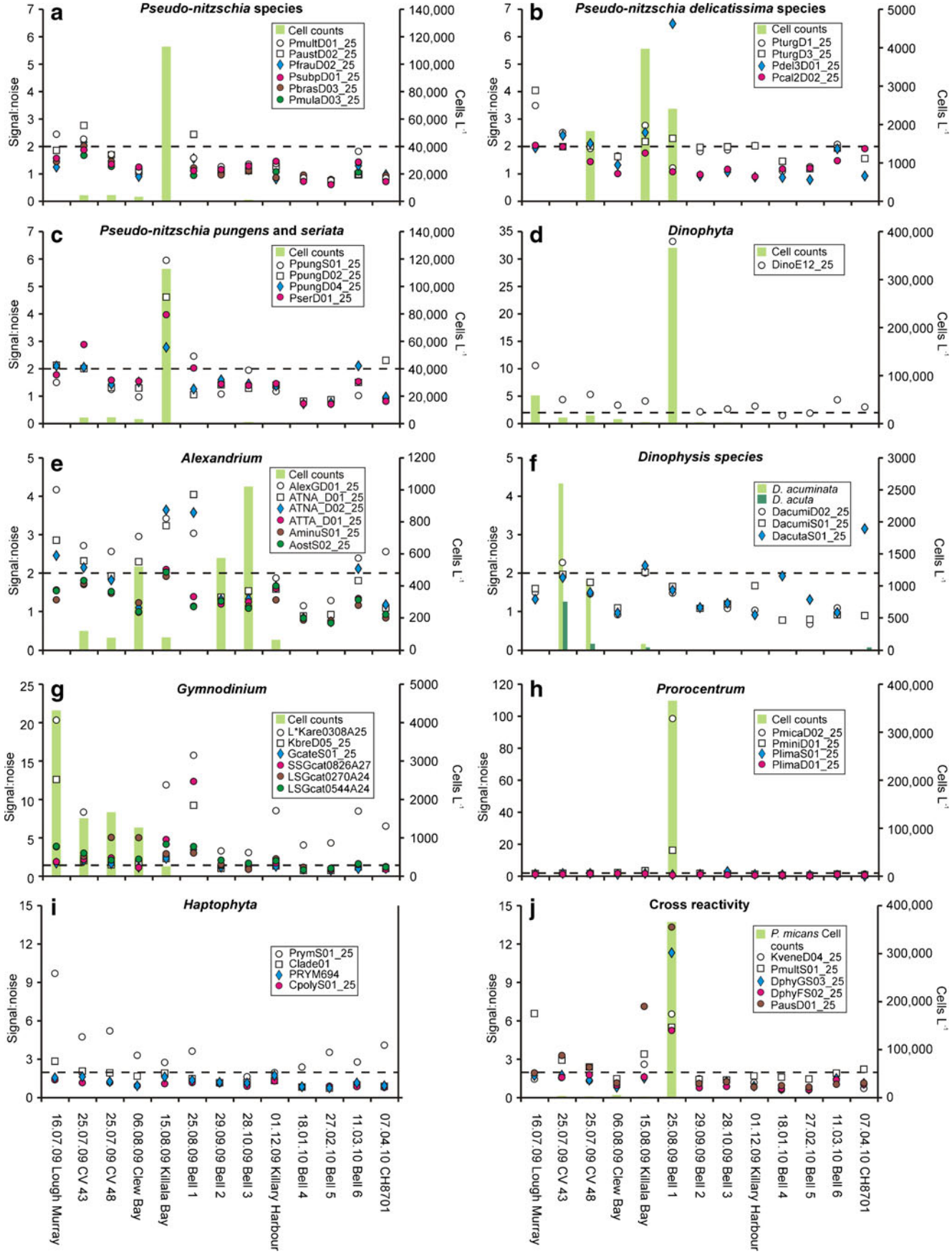
Scientific classification	Probe	*P. polylepis*		*P. parvum*	
		R^2 values	Intercept	R^2 values	Intercept
Eukaryotes	EukS_1209_25	0.91	2.02	0.95	1.66
Eukaryotes	EukS_328_25	0.48	3.74	0.96	2.26
Prymnesiophyta	PrymS01_25	0.83	2.88	0.98	2.83
Prymnesiophyta	PrymS02_25	0.88	2.63	0.97	2.44
Prymnesiophyceae	PrymS03_25	–	–	0.92	1.35
Prymnesium clade	Clade01	0.98	1.49	0.98	1.49
P. polylepis	CpolyS01_25	0.85	1.88	–	–
P. parvum	PRYM694	–	–	0.80	1.16
Probe considered unsatisfactory					
Eukaryotes	EUK1209	0.83	1.31	0.96	1.04
Eukaryotes	Euk328	0.97	1.43	0.33	1.03
Prymnesiophyceae	PrymS03_25	0.29	2.08	–	–
Prymnesium genis	PrymGS01_25	0.96	1.31	0.99	1.20
Prymnesium genis	PrymGS02_25	0.90	1.07	0.14	1.13
P. polylepis	CpolyS01_25	–	–	0.76	1.05
P. polylepis	CPOLY01	0.97	1.32	0.76	1.08
P. parvum	PRYM694	0.80	1.16	–	–

Pseudo-nitzschia delicatissma group were detected by LM in stations CV48, Killala Bay, Bell 1 and Bell 6 (maximum of 4000 cells l^{-1}). The probe Pdel3D01_25 which is specific for all *P. delicatissma* Clade1 and *P. multistriata* resulted in a positive microarray signal for all of the four samples. The probe also returned a false-positive result (detected by the microarray, but not present in the cell counts) for sample CV43. *P. turgiduloides* probes PturgD1_25 and PturgD3_25 were also highlighted on the microarray. Lough Murray showed false-positive readings for both these probes and another false-positive for PturgD1_25 in sample CV43. There were also some false-negatives for both *Populus turgiduloides* probes in sample CV48 but these values were very close to the threshold limit (1.90 and 1.97 s/n ratio, respectively), PturgD3_25 also had a false negative in sample Bell 6 (Fig. 6b).

In Fig. 6d, a comparison of the microarray probe signal with the total *Dinophyta* count in the water sample showed a good correspondence for the class level probe DinoE12_25. There were dinoflagellate species present in 11 out of 13 sampling stations. Bell 4 (18.01.10) and Bell 5 (27.02.10) were the only stations to have an absence of dinoflagellates which was reflected in the microarray result with signal-to-noise ratio values below 2 (Fig. 6d).

Alexandrium species were found to be present in eight of the 13 stations sampled and cell numbers ranged from 64 to 1,020 cells l^{-1} (Fig. 6e). The genus level probe AlexGD01_25 gave a positive result for five of the eight samples containing *Alexandrium* cells (CV43, CV48, Clew Bay, Killala Bay, and Bell 6). The three remaining samples (Bell 2, Bell 3, and Killary Harbour) gave false-negative results; this may be because of the extraction method problems. Out of all the 13 samples, only three (Lough Murray, Bell 1, and CH8701) gave false-positive results for this genus level probe. The microarray results for species level probes *Alexandrium tamarense* North American (NA) and Temperate Asian (TA) ribotypes, *Alexandrium minutum* and *Alexandrium ostenfeldii* are also presented in Fig. 6e. There were positive microarray signals for *A. tamarense* (NA) probe ATNA_D01_25 in stations CV43, Clew Bay, and Killala Bay, however, there were false-negative results in stations CV48, Bell 2, Bell 3, Killary Harbour, and Bell 6. Lough Murray and Bell 1 producing false-positive results. The second *A. tamarense* (NA) probe ATNA_D02_25 showed a positive signal-to-noise ratio >2 in four of the eight samples (CV43, Killala Bay, Killary Harbour, and Bell 6). However, again Lough Murray and Bell 1 showed a false-positive result for this species. The reason for these false positives may be cross reactivity with other phytoplankton genera; there was a high number of *Scrippsiella* sp. present in Lough Murry and a high number of *P. micans* present in Bell 1, which are also grouped to the Dinophyta class. *A. tamarense* (TA) probe ATTA_D01_25 only gave a >2 signal-to-noise ratio

Fig. 6 Comparisons between light microscopy counts and 2nd generation microarray results for field samples taken during 2009–2010. Results are presented for **a**–**c** *Pseudo-nitzschia* spp., **d** the taxonomic class probe for the *Dinophyta*, **e** genus level and five species level probes for *Alexandrium*, **f** *D. acuta* and *D. acuminata* probes, **g** probes for various *Gymnodinium* forms (see Table 2 for further details), **h** *Prorocentrum* spp., **i** haptophyte probes: no light microscopy counts were available for this group, **j** examples showing cross-reactivity of various probes against counts of *P. micans*


a
Pseudo-nitzschia species
Cell counts
PmultD01_25
PaustD02_25
PfrauD02_25
PsubpD01_25
PbrasD03_25
PmulaD03_25
b
Pseudo-nitzschia delicatissima species
Cell counts
PturgD1_25
PturgD3_25
Pdel3D01_25
Pcal2D02_25
c
Pseudo-nitzschia pungens and seriata
Cell counts
PpungS01_25
PpungD02_25
PpungD04_25
PserD01_25
d
Dinophyta
Cell counts
DinoE12_25
e
Alexandrium
Cell counts
AlexGD01_25
ATNA_D01_25
ATNA_D02_25
ATTA_D01_25
AminuS01_25
AostS02_25
f
Dinophysis species
D. acuminata
D. acuta
DacumiD02_25
DacumiS01_25
DacutaS01_25
g
Gymnodinium
Cell counts
L*Kare0308A25
KbreD05_25
GcateS01_25
SSGcat0826A27
LSGcat0270A24
LSGcat0544A24
h
Prorocentrum
Cell counts
PmicaD02_25
PminiD01_25
PlimaS01_25
PlimaD01_25
i
Haptophyta
PrymS01_25
Clade01
PRYM694
CpolyS01_25
j
Cross reactivity
P. micans Cell counts
KveneD04_25
PmultS01_25
DphyGS03_25
DphyFS02_25
PausD01_25
Signal:noise
Cells L-1
16.07.09 Lough Murray
25.07.09 CV 43
25.07.09 CV 48
06.08.09 Clew Bay
15.08.09 Killala Bay
25.08.09 Bell 1
29.09.09 Bell 2
28.10.09 Bell 3
01.12.09 Killary Harbour
18.01.10 Bell 4
27.02.10 Bell 5
11.03.10 Bell 6
07.04.10 CH8701

in Killala Bay sample. All the other samples showed a signal-to-noise ratio <2, not a surprising result as this ribotype is absent in Irish coastal waters (Lilly et al. 2002). *Alexandrium catenella* isolates that are grouped with the *A. tamarense–A. catenella–Alexandrium fundyense* species complex have been reported present in the Thau Lagoon of the French Mediterranean coast and are of the Japanese ribotype of Temperate Asian clade (Lilly et al. 2002; Masseret et al. 2009).They are also highly unlikely to be present in field samples from Ireland. *A. minutum* (AminuS01_25) and *A. ostenfeldii* (AostS02_25) probes showed a weak signal across all samples; however, *A. minutum* is the most likely species present in the field samples especially in the North Channel Cork Harbour sample CH8701, which has been known for its contamination of shellfish with paralytic shellfish poisoning toxin (Touzet et al. 2007, 2011). In general, the *Alexandrium* microarray results correlated poorly with the cell counts, with numerous false positives and negatives. Further adaptations will be required to improve the *Alexandrium* probe set in the third generation chip.

Dinophysis sp. cells were observed in four field samples. *Dinophysis acuta* (range, 40–760 cells l^{-1}) was present but the signal-to-noise ratio did not exceed 2 in CV43 and CV48 and relationship with field samples was weak (Fig. 6f). Only in Killala Bay and CH8701 samples was the signal-to-noise ratio >2 with the species specific probe DacutaS01_25, even though the cell numbers were below that of CV43 and CV48. It is possible that there were larger numbers of cells on the filters than in the Lugol's samples because of the greater volume filtered for RNA extraction. *Dinophysis* have been known to escape detection with LM because of very low densities. This does not negate from their potential to cause diarrheic shellfish poisoning events, which is the one of the main causes of closures of Irish mussel farms during late spring and early autumn (Raine et al. 2010). *Dinophysis acuminata* was present in CV43, CV48 and Killala Bay (range, 40–1,840 cells l^{-1}) and detected on the microarray via the two species specific probes DacumiS01_25 and DacumiD02_25. DacumiD02_25 only gave a signal-to-noise ratio above 2 in the CV43 sample, whilst DacumiS01_25 probe only gave a signal-to-noise ratio above 2 in the Killala Bay sample. Sample CV48 recorded a false-negative result for both probes. Dinophysiaceae (*Dinophysis+Phalacroma*) family DphyFS02_25 and genus (DphyGS03_25) probes did not correlate well either and gave very low signals or may have been cross reacting with other species, such as the dominating *P. micans* bloom in Bell Harbour 1 (Fig. 6j).

Gymnodinium species were detected by LM in seven of 13 stations sampled. *Karenia* genus level probe (L*Kare0308A25) gave a signal-to-noise ratio >2 for seven samples (Lough Murray, CV43, CV48, Clew Bay, Killala Bay, Bell 1, and Bell 6); however, there was also a false-positive result for all the remaining six samples. The other *Karenia* genus level (KbreD05_25) probe gave positive microarray signal for four samples (Lough Murray, CV43, Killala Bay, and Bell 1) containing *Gymnodinium* species. However CV48, Clew Bay and Bell six showed false-negative results. The elevated signal in the Bell 1 sample may be caused by *Gymnodinium* cells being overlooked because of the extensive P. *micans* bloom (Fig. 6h). There also may be some cross reactivity with this bloom event along with *Gymnodinium catenatum* species probe (SSGcat0826A27) and a number of other genera probes shown in Fig. 6j. Two other *G. catenatum* species level probes LSGcat0270A24 and LSGcat0544A24 showed in general a good correlation with LM counts. There are however two exceptions with both these probes as there was a false-positive result from Killary Harbour and a false negative from Bell 6 which may be because of the low numbers recorded. *Karenia mikimotoi* is one of the most frequently observed red tide causing dinoflagellates in the North Atlantic and has been known to occur all along the Irish coastline (Raine et al. 2001), being responsible for the major mortality of benthic and pelagic marine organisms which occurred in 2005 (Silke et al. 2005).

An extensive bloom of *P. micans* (360,000 cells l^{-1}) was observed in Bell Harbour on the 25th August 2009. This was reflected in the microarray results. However, *P. micans* was also detected in six other field samples, with cell numbers ranging from 64 to 4,700 cells l^{-1}. Only the Killala Bay sample gave a microarray signal above the signal-to-noise ratio value of 2. All the other five samples (CV43, CV48, Clew Bay, Bell Harbour 3, and Killary Harbour) gave a low signal and this species was not detected by the microarray (false negatives). This may have resulted from the low cell numbers or RNA extraction protocol not being stringent enough in relation to breaking up of the cells. There was a substantial difference between the *P. micans* bloom event and the other samples containing cell numbers below 5,000 cells l^{-1}. However, the counts were well corroborated by the microarray signal data (Fig. 6h). The *Prorocentrum minimum* (PminiD01_25) probe also gave a signal-to-noise ratio above 2 in two samples from Killala Bay and Bell Harbour 1. This organism may have been present in the samples but was not counted in the LM counts. *Prorocentrum lima* (PlimaS01_25 and PlimaD01_25) was not detected in the cell counts, confirming the microarray results.

Haptophyta results were difficult to analyse because of the inability of identification to species level in preserved samples using LM. During LM analysis, they are mostly recorded as unidentified microflagellates and were most likely grouped along with a number of various other microflagellate species. Therefore, comparing cell counts with the microarray can become troublesome and may be inaccurate without the aid of more skilled techniques such as electron microscopy (Fig. 6i).

Conclusion

It is apparent that some probes were working better than others and this required the testing of RNA amounts that could be

detected by the microarray for each individual probe on the chip. This cutoff of detection can be seen through the hierarchical probe set from higher group probes which is producing a higher signal right down to species level probes which can produce weaker signals. This is why calibration curve were performed for each individual probe converting microarray signal to cell numbers.

In general, the cell count results have supported the microarray data. However, there were a few false-positive results detected by the microarray, possibly indicating the presence of species that were not recorded in light microscopy cell counts. This was most likely caused by the larger volume used for filtration (~0.2–1 L) compared to the small volume taken for cell counts (50 ml), or else an inability to identify cells to species level by light microscopy alone. Unspecific binding is another issue that was particularly apparent when comparing the *P. micans* counts with false-positive microarray signals from the Bell Harbour 1 (August 2009) sample (Fig. 6j). Although these are just a few comparisons of light microscopy counts with microarray results in field samples, all of year 1 samples were hybridised to the second generation chips and in many cases had a low labelling efficiency, which can be a direct reflection of the quality of the extracted RNA, where crossover of NaCl, TRIS, EDTA, or proteins may have occurred. These methodological issues will be addressed in the third generation microarray. Adaptations to the RNA extraction protocol, such as the addition of longer bead beating to ensure successful breaking of cells, the introduction of RNA revised clean-up steps to improve labelling efficiency, and improvements to hybridisation protocol, should decrease the number of false positives, reduce cross reactivity and increase specificity and sensitivity.

The aim of the MIDTAL project is to provide a new method to support toxic algal monitoring, to contribute to human health and common fisheries policies. These first field results indicate that there remains further development work to be done but point towards the potential successful development of a "universal" HAB microarray. Further adaption and optimisation of existing rRNA probes to a third generation microarray are still ongoing.

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